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The 12S adenoviral E1A protein immortalizes avian cells and interacts with the avian RB product

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Quail cells were immortalized for the first time by using retroviruses expressing the 12S adenoviral E1A gene. In these cells, interaction between the 12S E1A product and the quail RB protein was shown, suggesting that the 12S adenoviral E1A product works in avian cells through similar biochemical pathways as in mammalian cells by interacting and inactivating host cellular proteins, including the RB product. These results confirm that the RB product exhibits a universal function among higher vertebrates in controlling cellular growth and tumor progression.

Introduction

Tumor progression is a multistep process that involves activation of proto-oncogenes and inactivation of suppressor genes (Bishop, 1991). Inactivation of the function of suppressor genes occurs as the result of either gene rearrangement, deletion, mutation or inactivation of the protein by products of tumor viruses. The two most extensively investigated antioncogene proteins are the RB and p53 products. The RB product is inactivated by binding to the large T protein of the SV40 virus (DeCaprio *et al.*, 1988), the E1A product of adenovirus (Whyte *et al.*, 1988) and the E7 protein of human papillomavirus (Münger *et al.*, 1989). The p53 product is inactivated by the SV40 large T antigen (Lane & Crawford, 1979), the E1B of adenovirus (Sarnow *et al.*, 1982) and E6 of human papillomavirus HPV-16 and -18 (Werness *et al.*, 1990), usually resulting in immortalization of the cells. Thus, these viral products provide helpful experimental tools to mimic natural inactivation of antioncogenes in cell culture and investigate the role of this process in tumor progression.

Immortalization by oncogenes from DNA tumor virus has been widely described in mammalian species (Linder & Marshall, 1990) but never in avian species, in which immortalization efficiency is very low. On the other hand, no similar DNA tumor virus has so far been described in these species. Since avian cells have been widely used to investigate transformation by oncogenes, mostly of retrovirus origin, it would be helpful to develop an experimental approach to analyse the contribution of antioncogenes in tumor transformation in these cells. For this purpose, we have constructed avian retrovirus vectors that express the 12S product of the human adenovirus E1A gene. The availability of a powerful avian transcomplementing cell line (Cosset *et al.*, 1990) made it feasible to develop efficient helper-free recombinant retroviruses.

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Results

Properties of TXN12S retroviruses encoding the 12S adenoviral E1A gene

In the two constructs TXN5'12S and TXN3'12S, the 12S product is translated from subgenomic mRNAs from its natural initiation codon as depicted in Figure 1. The neomycin phosphotransferase product, allowing selection of virus-infected cells by the drug G418, is translated from either genomic mRNA (TXN5'12S) or subgenomic mRNA (TXN3'12S). After transfection into the ISOLDE packaging cell line (Cosset *et al.*, 1990), helper-free viruses were obtained. The titers of

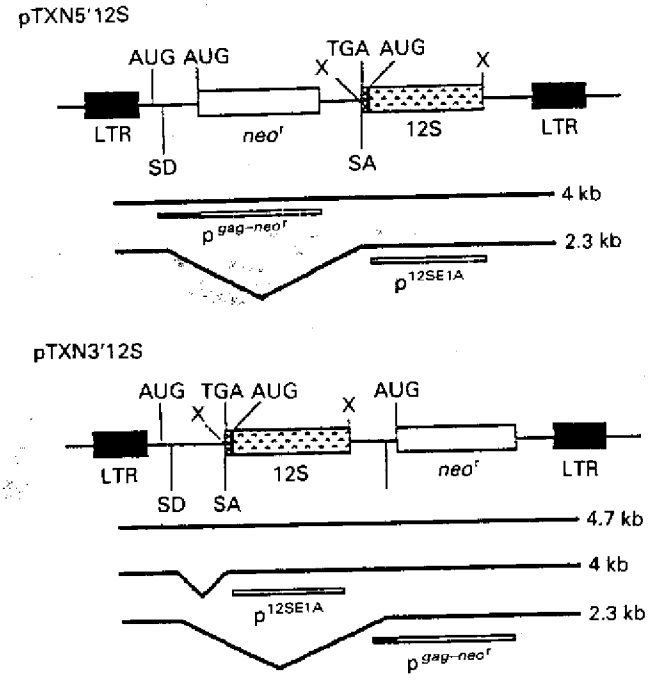

Figure 1 Structure of retrovirus vectors encoding the 12S adenovirus E1A sequence. The figure shows the different mRNA transcripts from which the 12S E1A and *neo*^{r} products are translated. LTR, avian erythroblastosis virus long terminal repeat; SD, splice donor site; SA, splice acceptor site; X, XbaI

the TXN12S vector stocks varied from 10^3 to 10^4 viruses per ml as assessed by their ability to confer G418 resistance on chicken embryo fibroblasts (CEFs).

Establishment of quail cell lines

Plated quail embryo brain and liver cells form relatively homogeneous populations that exhibit an epithelioid morphology with extensive cell–cell contacts. Such primary cells continue to grow for 4–5 days, enabling retroviral infection before reaching confluence. After 2–3 passages, cultures of liver cells usually vanish, whereas most brain cells begin to exhibit the morphology of senescent cells (Figure 2d).

Infection with TXN12S retroviruses followed by G418 selection allowed us to obtain many clones of growing liver and brain cells. Only clones that were sufficiently furnished and looked vigorous were individually isolated; others were pooled (Table 1). Outgrowths of cell lines were usually obtained after a crisis period of around 2 months. Although it was possible to obtain clones after infection with the control virus TXN3′ expressing only the neomycin phosphotransferase gene, such cells were unable to expand.

The TXN12S-infected cells were continuously cultured by serial passages thereafter for more than 1 year. The QBr3, QBrA and QHep1 cell lines have currently reached 54, 52 and 34 passages respectively, which corresponds to 90–120 generations. The cells exhibit an epithelioid morphology (Figure 2a–c) and cover the culture plates as pavements. The QBr cell lines differ from the QHep line in that they show tighter contact with neighboring cells when confluent. Morphology and transmission electron microscopy of these cells suggest that they have an epithelial origin (data not shown), but these cells are positive for the expression of the vimentin and negative for cytokeratins 8 and 18. Nevertheless, cytokeratins are not always expressed by epithelial cells *in vitro*. In culture, these cells can also become vimentin positive, which is usually an *in vivo* fibroblastic characteristic (Wiehle *et al.*, 1990).

To check if these cells infected by the TXN12S viruses did exhibit features of transformed cells, we tested their ability to develop colonies in soft-agar culture as described previously (Gandrillon *et al.*, 1987). As a positive control, we used LMH cells, which are derived from a liver epithelial cell line established

Table 1 Infection and immortalization of quail embryo cells using TXN3′12S and TXN5′12S retroviruses

Origin of cells	*Virus*	*No. of isolated clones*	*Immortalized cell lines*
Brain	TXN5′12S	13/25* + two pools	One pool = QBrA
	TXN3′12S	8/30* + one pool	One clone = QBr3
Liver	TXN5′12S	4/6* + one pool	One clone = QHep1
	TXN3′12S	One pool	

*No. of isolated clones/no. of numbered clones

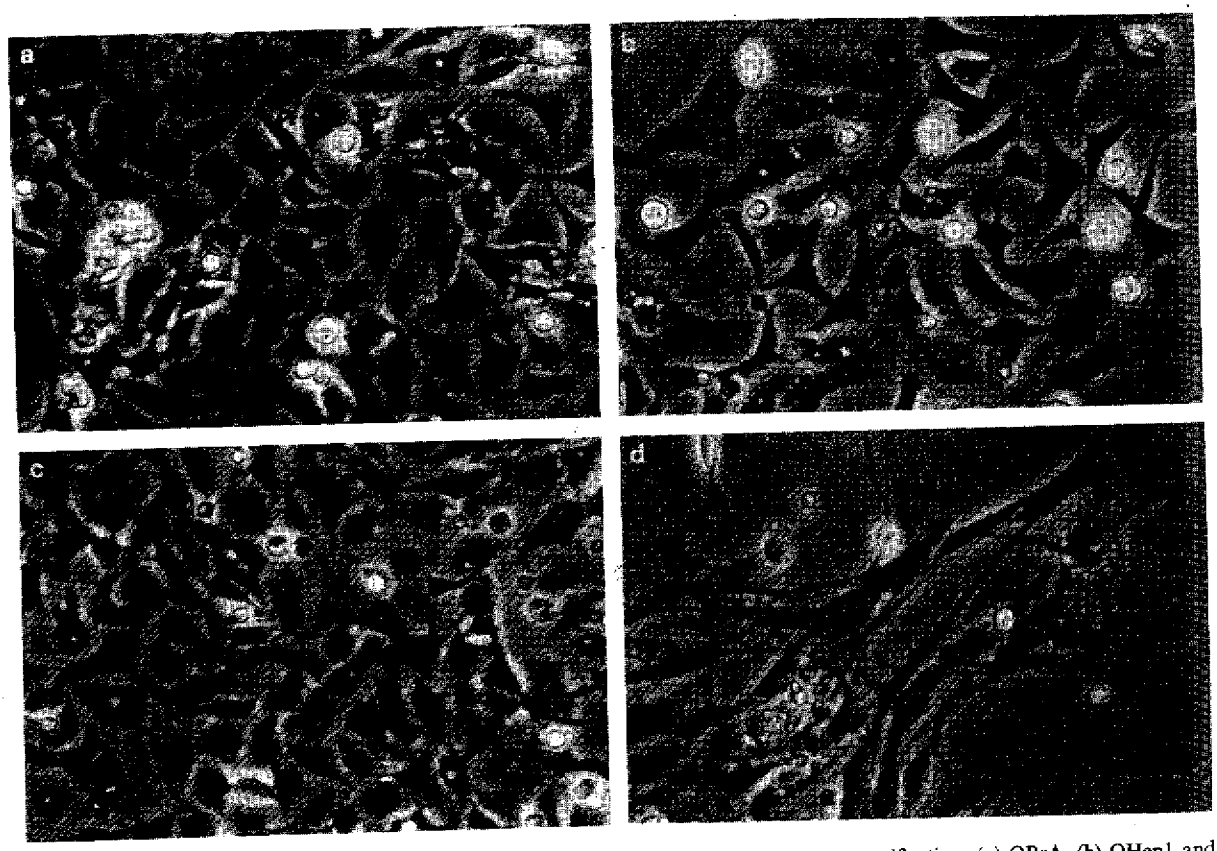

Figure 2 Morphology of QBr and QHep cell lines. Phase-contrast photographs, 500 × magnification. (**a**) QBrA, (**b**) QHep1 and (**c**) QBr3 at passage 52, 34 and 54 respectively. Non-infected brain cells are shown at passage 11 (**d**)

by a chemical carcinogen treatment in chicken *in vivo* (Kawaguchi *et al.*, 1987). Whereas these cells gave rise to large colonies (Figure 3a) at a high frequency (5000–6000 colonies for 10^5 cells), the QBr3 and QBrA did not grow in soft agar (Figure 3b and c). Only the QHep1 cells developed very small colonies at a low frequency (50–200 colonies for 10^5 cells) (Figure 3d).

Expression of the 12S adenoviral E1A gene

As an initial molecular characterization of the different cell lines, we looked for the E1A-12S protein *in situ*, by an indirect immunophosphatase alkaline reaction using the monoclonal antibody M73, which specifically recognizes the E1A product (Harlow *et al.*, 1985). The 12S protein was detected in the nucleus of the different cell cultures (Figure 4).

Immunoprecipitation of cell lysates with the monoclonal antibody M73 was performed as described by Harlow *et al.* (1986) to confirm the expression of the 12S product. As a positive control, we used the adenovirus 5-transformed human embryonic kidney cell line 293 (Graham *et al.*, 1977). The antibody PAb 416 (Harlow *et al.*, 1981), which recognizes SV40 large T, was used as a control antibody. As a negative control, QT6 cells, which are from a quail fibroblast cell line transformed by carcinogen (Moscovici *et al.*, 1977), were infected with TXN5′ retrovirus (Benchaibi *et al.*, 1989) expressing only the neomycin phosphotransferase gene. Lysates from these cells were also immunoprecipitated with the M73 antibody. The results in Figure 5 show that the 12S E1A is not detected in QT6 cells but clearly found in the three

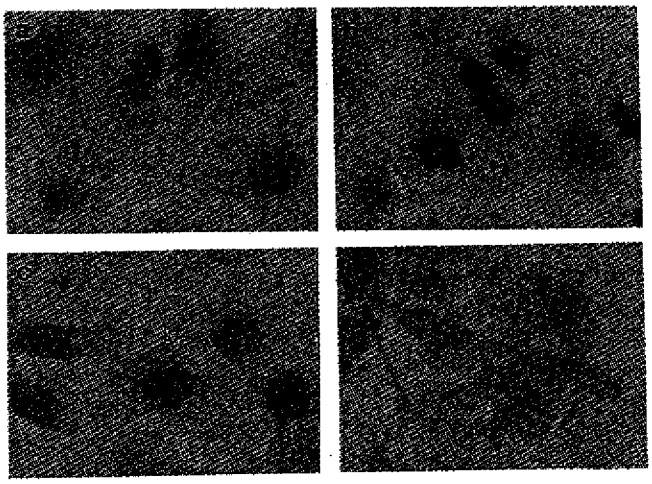

Figure 4 Immunostaining for 12S E1A protein in the (a) QBrA, (b) QBr3, (c) QHep1 and (d) QT6 TXN5′ cell lines. 1000 × magnification

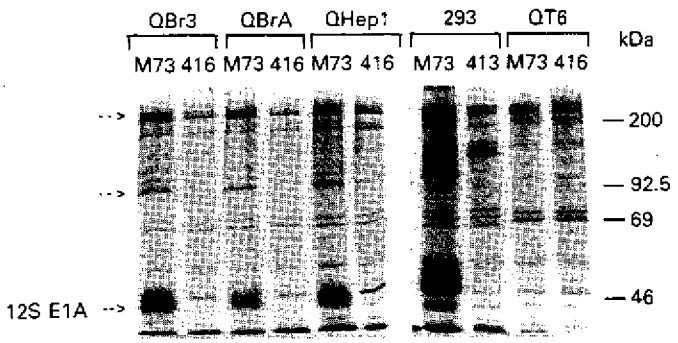

Figure 5 Immunoprecipitation of the 12S E1A protein. The immunoprecipitation of 12S E1A protein from QBr3, QBrA and QHep1 cells at passage 28, 23 and 17 respectively is shown. Lysate from 293 cell line was used as a positive control. Cells were labeled with [^{35}S]methionine for 4 h and equal numbers of trichloroacetic acid-precipitable counts were used for immunoprecipitation with the anti-E1A monoclonal antibody M73 as described previously. As a control, lysates from the different cell lines were immunoprecipitated with the monoclonal antibody PAb 416, which recognizes the large T SV40 product. Samples were electrophoresed on 8% polyacrylamide gel

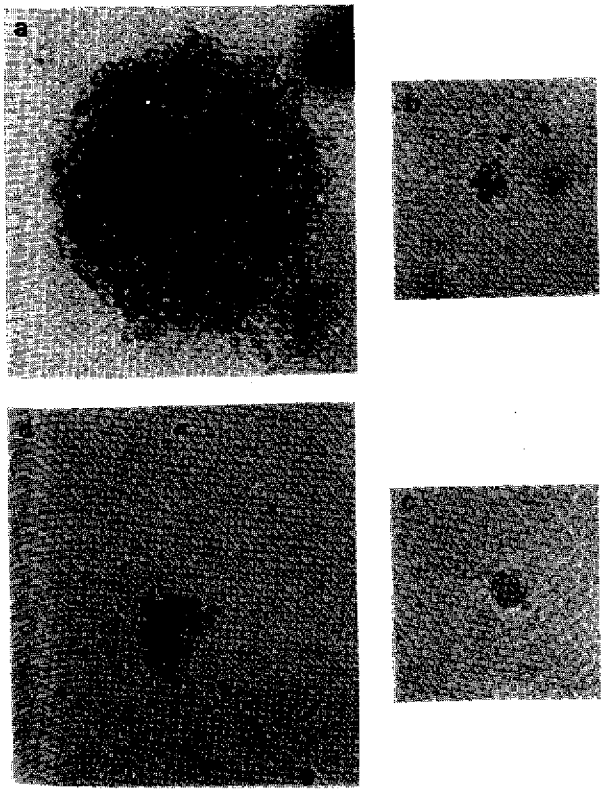

Figure 3 Colony formation in agar: (a) LMH, (b) QBrA, (c) QBr3 and (d) QHep1. QBrA, QBr3 and QHep1 cells were tested at passages 26, 31 and 19 respectively. 120 × magnification

TXN12S-induced quail cell lines, albeit at a slightly lower level than in the 293 cell line. It was noteworthy that these three cell lines did not express similar levels of the 12S protein. These differences could result from different strategies of expression of the 12S product by the viruses TXN3′12S and TXN5′12S (Figure 1).

Association of the 12S adenoviral E1A protein with the quail RB protein

In mammalian cells expressing the 12S product, immunoprecipitation with the monoclonal antibody M73 revealed the presence of many proteins associated with the 12S protein: predominantly the 105-kDa RB protein and a 300-kDa protein (Harlow *et al.*, 1986). To check if the quail RB gene product is complexed with the 12S protein in the QBr and QHep cells, we performed an immunoprecipitation of cell lysates with

the M73 monoclonal antibody followed by transfer to a nitrocellulose membrane and Western analysis using the purified monoclonal anti-RB antibody 14001A (Pharmingen). This procedure revealed the expected 105-kDa RB product in the 293 cell line (Figure 6a). In the quail cells, we barely detected a unique band at a slightly lower size of 95 kDa. To confirm the nature of this protein, we tested another monoclonal antibody on the QBr3 cell lysate. The XZ55 antibody (Hu *et al.*, 1991) directed against a different epitope of the human RB product revealed the same band as the 14001A antibody (Figure 6b). The weakness of the signal in avian cells as compared with the 293 cell line presumably results from poor affinity of the antibodies against a distantly related RB product. Since this protein is revealed by two monoclonal antibodies against RB and is not found in lysates of QT6 cells not expressing the 12S product or immunoprecipitated by the PAB 416 antibody (Harlow *et al.*, 1981), it is likely that it corresponds to the quail RB protein or a protein closely related to RB. These data show therefore that in established quail cell lines the 12S E1A product is complexed with RB or an RB-related product. Immunoprecipitation of quail cell lysates using M73 antibody also revealed interaction with a protein of about 300 kDa (Figure 5, arrow), which could be related to the 12S-linked p300 protein as described previously in human cells (Harlow *et al.*, 1986). It was not possible to detect other specific proteins known to complex with 12S E1A product in mammal cells such as p60, p107 and p130 proteins (Whyte *et al.*, 1989; Herrmann *et al.*, 1991).

Effect of transforming growth factor beta (TGF-β) on the growth of 12S-expressing cells

The growth inhibitory effect of TGF-β1 on some cells has been shown to be abrogated by the 12S E1A product (Missero *et al.*, 1991). These observations and some other works (Laiho *et al.*, 1990; Pietenpol *et al.*, 1990) suggest that the RB product and/or other products such as p300 (Missero *et al.*, 1991) might be involved in the pathway that mediates this growth inhibition. To check if the TXN12S viruses could induce similar effects in avian cells, we compared the effect of TGF-β1 on the growth of chicken embryo fibroblasts infected with either the control virus TXN5′ or the TXN12S viruses. We did not perform this test on the QBr and QHep cells since we did not have normal counterparts of these cells that might be used as controls. As shown in Figure 7, TGF-β1 strongly inhibited the growth of CEFs infected by TXN5′, but had no effect on the growth of CEFs infected by either TXN5′12S or TXN3′12S.

Discussion

Spontaneous immortalization of cells in culture is a rather rare event that can occur at different efficiencies in various animal species. For example, it is more efficient in mouse than in human cells. It is almost impossible in chicken or quail cells. However, some avian cells have been established after transformation *in vivo* by carcinogens (Moscovici *et al.*, 1977; Kawaguchi *et al.*, 1987) or *in vitro* by avian leukemia retroviruses (Beug *et al.*, 1982). In the latter case, establishment of leukemia cells is infrequent and requires a long crisis period and relies presumably on inactivation of the p53 gene or p53 product (Ulrich *et al.*, 1992). Susceptibility to immortalization of such avian cells might depend on genetic background, since with our quail and chicken flocks we have been unable to derive cell lines from virus-transformed cells, although these cells could be kept alive for long periods of time in growth crisis.

DNA tumor viruses have been widely used to immortalize mammalian cells, but no such viruses with similar properties in avian cells have been described. We then constructed avian retrovirus vectors carrying the E1A-12S gene, an adenovirus gene known to induce immortalization of mammalian cells (Cone *et al.*, 1988; Emami *et al.*, 1989; Wiehle *et al.*, 1990). We show here that these viruses are efficient in immortalizing quail cells from liver and brain, whereas no immortalization can result from infection by control retroviruses carrying no oncogene. Immortalization does not result from any effect of helper virus gene products since our retrovirus vectors were produced free of helper virus through an avian helper cell line (Cosset *et*

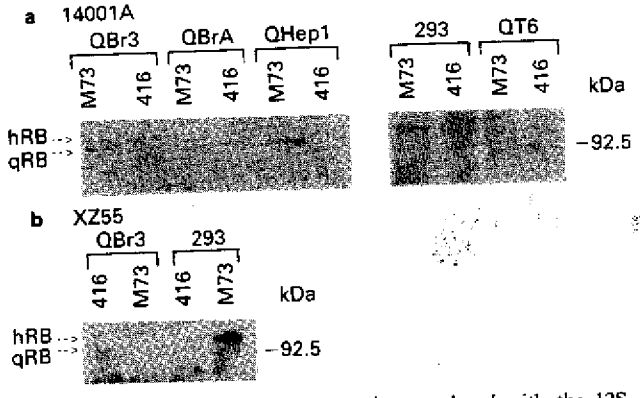

Figure 6 Western blot of RB protein complexed with the 12S E1A protein. Cells of QBr, QHep and 293 cell lines were first immunoprecipitated with the anti-E1A monoclonal antibody M73. Samples were electrophoresed on 8% polyacrylamide gel, then transferred to nitrocellulose membrane. Anti-human RB monoclonal antibodies 14001A (Pharmingen) (**a**) and XZ55 (Hu *et al.* 1991) (**b**) were used to reveal the blotted products

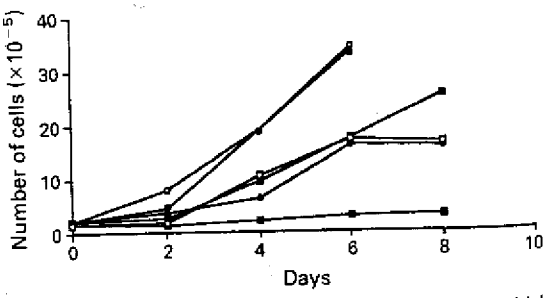

Figure 7 Effect of TGF-β1 on TXN12S-infected CEFs (chicken embryo fibroblasts): 2×10^5 TXN12S-infected and selected CEFs were seeded in 35-mm dishes with or without TGF-β1 (10 ng ml^{-1}). The medium was changed every 2 days. CEFs infected with TXN5′ virus expressing only the neomycin phosphotransferase gene were used as controls. Each point represents an average of two different dishes. □, TXN5′12S + TGF-β; ◆, TXN5′12S; ●, TXN3′12S + TGF-β; ○, TXN3′12S; ■, TXN5′ + TGF-β; □, TXN5′

al., 1990). Since the E1A-12S product was detected in the nucleus of the immortalized cells, it is then clear that it is this product that is directly responsible for the immortalization. Note that, as in mammals, the avian cells harboring the E1A-12S product do not exhibit transformation features such as the ability to develop colonies in soft agar.

In mammalian cells, the E1A-12S product binds several proteins, including the product of the RB antioncogene. It is assumed that the complexed RB product is no longer able to prevent proliferation and thereby promotes immortalization. We show that in immortalized quail cells the E1A-12S protein is complexed with a product that is recognized by two monoclonal antibodies specific to human RB. This product is not revealed in quail cells not expressing the E1A-12S product. These data show therefore that the avian and mammalian RB products are highly conserved and display binding functions identical to other products such as E1A-12S. As in mammalian cells, the E1A-12S relieves the avian cells from growth inhibition induced by TGF-β1, a process supposed to involve an intracellular pathway mediated by RB (Laiho *et al.*, 1990; Pietenpol *et al.*, 1990; Missero *et al.*, 1991). Taken together, all these data show that the structure and function of RB have been highly conserved between birds and mammals.

Interactions between E1A-12S and RB should be important for immortalization of avian cells but might not be sufficient. Indeed, the cell lines we isolated were all obtained after a growth crisis period, which suggests that other events are important for immortalization. In fact, we can imagine that E1A-12S gene expression is a first event inducing uncontrolled cell proliferation that would predispose some cells to mutations or rearrangements in regulatory genes, thereby enabling immortalization.

The TXN12S retroviruses therefore constitute powerful experimental tools to inactivate the RB protein in avian cells and thereby analyse the contribution of this antioncogene product to the control of cell proliferation and tumor progression and its role in the development of the embryo.

Materials and methods

Construction of recombinant retroviruses

The 12S adenoviral E1A cDNA from plasmid pE1A-12S (Gilardi & Perricaudet, 1984) was modified using synthetic oligonucleotides to obtain a sequence containing only the coding region. This cDNA was inserted downstream of the splice acceptor region of the *v-src* oncogene from Rous sarcoma virus (RSV) derived from plasmid pGAS24 (a gift from M. Castellazzi) having a stop codon and a splice acceptor site allowing initiation of the transcription to the original initiation codon of the 12S adenoviral E1A gene. The whole fragment was then inserted at the XbaI site of the retroviral vectors TXN5′ and TXN3′, which carry the neomycin phosphotransferase (*neo*^{r}) gene (Benchaibi *et al.*, 1989) to give vectors TXN5′12S and TXN3′12S respectively. The plasmids carrying the recombinant vector genomes were transfected into the ISOLDE packaging cell line (Cosset *et al.*, 1990). G418 was added 24 h later at a concentration of 200 μg ml^{-1}. Foci of resistant ISOLDE cells usually became visible after 10 days. The drug was then removed and the cells passaged in regular CEF medium. Viruses were usually collected from subconfluent cultures.

Primary cell culture and infection with recombinant retroviruses

Liver and brain tissues were obtained from 13–14-day-old quail embryos (Coturnix Coturnix Japonica from Cailles des Dombes). Organs were finely minced and then digested at 37°C for 5 min under stirring in 0.5 mg of trypsin per ml of Tris–glucose buffer (TG). This step was repeated until the digestion was complete. The suspension was next filtered through a sterilized gauze and centrifuged. The cells were plated at a density of $2-4 \times 10^6$ cells per 60-mm plate in Ham's F-10/199 (v/v) containing 4% fetal calf serum and 1% chicken serum. The next day, the medium was replaced by the same medium without serum to inhibit the growth of fibroblasts. Two days later, serum and growth factors [insulin (10 μg ml^{-1}), conalbumin (100 μg ml^{-1}), hydrocortisone (300 ng ml^{-1})] were added and infection with 200 μl of TXN12S or TXN virus stocks was performed. In some cases, one second round of infection was performed 1 day later to increase the number of infected cells. The day following the last infection, cells were selected with G418 (150 μg ml^{-1}). Resistant clones began to appear 15 days later and were isolated as soon as they were sufficiently furnished.

Colony-forming assay in soft agar

The colony-forming assay was performed as previously described (Gandrillon *et al.*, 1987). Colonies were observed 2 weeks later.

Immunostaining assay

Formaldehyde-fixed cultures were stained by an indirect immunophophatase technique using the M73 monoclonal antibody against the E1A protein (Harlow *et al.*, 1985).

Immunoprecipitation

Equal numbers of cells (5×10^6) were labeled for 4 h with 400 μCi of [^{35}S]methionine (>1000 Ci mmol^{-1}; Amersham) in 100-mm plates containing 2 ml of Dulbecco's modified Eagle medium (DMEM) without methionine. The monolayers were washed once with TNE (Tris 50 mM, sodium chloride 150 mM, EDTA 1 mM) and lysed with 1 ml of lysis buffer (Hepes pH 7.0 50 mM, sodium chloride 250 mM, NP-40 0.1%, EDTA 5 mM, PMSF 1 mM) for 30 min on ice. Equal trichloroacetic acid-precipitable counts were immunoprecipitated with the monoclonal antibody M73 as described by Harlow *et al.* (1985). A monoclonal antibody directed against T antigen of SV40 (PAb 416) (Harlow *et al.*, 1981) was used for control immunoprecipitations. Samples were electrophoresed on 8% polyacrylamide gels.

Immunoblotting

Immunoblotting was performed after transfer to a nitrocellulose membrane of samples immunoprecipitated with the M73 monoclonal antibody. Western analyses were undertaken with the anti-human RB monoclonal antibodies 14001A (Pharmingen) and XZ55 (Hu *et al.*, 1991). Revelation was performed using NBT (nitroblue tetrazolium)/BCIP (bromochloroindolyl phosphate) or chemoluminescence (Amersham).

TGF-β assay

2×10^5 TXN12S-infected and selected CEFs were seeded in 35-mm dishes with or without TGF-β1 (Genzyme) (10 ng ml^{-1}). The medium was changed every 2 days. CEFs infected with TXN5′ virus expressing only the neomycin phosphotransferase gene were used as controls.

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